Stimulation of Apolipoprotein E Secretion in Human Hepatoma Hep G2 Cells by a Cyclic Acylpeptide, N-4909

Sir:

Apolipoprotein E (apo E) is mainly produced in the liver and secreted into plasma as a major component of several classes of lipoproteins. Plasma clearance of apolipoprotein B (apo B) containing lipoproteins such as very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), and low density lipoprotein (LDL) is regulated by apo E which is capable of binding to apo B, E receptor and putative chylomicron remnant receptors[1].

Two separate studies showed that intravenous injection of apo E causes a marked decrease of plasma cholesterol levels in hyperlipidemic rabbits[2,3]. Another study revealed that apo E prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits[4]. Data from these studies suggested that stimulators of apo E secretion from the liver may show hypolipidemic and antiatherogenic activities through increasing the apo E levels in plasma. Therefore, we have decided to search for stimulators of apo E secretion from human hepatoma Hep G2 cells which still exhibit many differentiating functions of human parenchymal cells[5].

During our screening for stimulators of the apo E secretion from microbial fermentation broths, we isolated an active substance N-4909 from the culture broth of *Bacillus* sp. No. 4691. Structure elucidation studies of N-4909 demonstrated that the compound was identical with a subcomponent of isohalobacillin (Fig. 1), which had been isolated from *Bacillus* sp. A1238 as an inhibitor of acyl-CoA: cholesterol acyltransferase (ACAT)[6]. In this paper, we describe the isolation of N-4909, its identification as the subcomponent of isohalobacillin and the biological activity of N-4909. We also determined the stereochemistry of the β-oxyacyl residue of N-4909, which had remained to be clarified.

Bacillus sp. No. 4691 was cultivated in three 500-ml Erlenmeyer flasks each containing 100 ml of a medium consisting of soluble starch 1.0%, molasses 1.0%, polypeptone 1.0% and beef extract 1.0% (pH 7.2 before sterilization). These flasks were shaken on a rotary shaker (220 rpm) for 48 hours at 28°C and then transferred into a 30-liter jar fermenter containing 15 liters of the production medium consisting of soybean meal 1.5%, dry yeast 0.2% and $CaCO_3$ 0.4%. The fermentation was carried out at 28°C for 48 hours under aeration of 20 liters/minute and agitation speed of 250 rpm. The cell pellet obtained from the fermentation broth was extracted with methanol (5 liters) and the extract was filtered. The filtrate was diluted with 5 liters of water and passed through a Diaion HP-20 column (3 liters). The column was washed with 6 liters of 50% methanol and the active material was eluted with 6 liters of methanol. The eluate was concentrated under reduced pressure to an oily residue, which was dissolved in methanol and applied to a silica gel column (500 ml). The column was washed with one liter of chloroform - methanol (95 : 5) and the active material was eluted with one liter of chloroform - methanol (80 : 20). This fraction was concentrated under reduced pressure to give a brownish powder, which was redissolved in methanol. The solution was subjected to reverse phase HPLC under the following conditions: column, Prep ODS (30 × 250 mm); mobile phase, methanol - H_2O - trifluoroacetic acid (95 : 5 : 0.05); flow rate, 30 ml/minute; detection, UV 222 nm. Under these conditions, the peak eluted at 15.5 minutes was collected to give a white powder of N-4909 (550 mg).

The molecular formula of N-4909 was determined to be $C_{53}H_{94}N_8O_{12}$ by the HR-FAB mass spectrum. The physico-chemical properties (Table 1) of N-4909 were in good agreement with the reported data for isohalobacillin[6]. These results indicated that N-4909 was one of a subcomponents of the isohallobacillin complex, having a different β-oxyacyl group in the molecule. To determine the structure of β-oxyacyl group of N-4909, methanolysis of the compound was carried out with 12 N-HCl - methanol (3 : 7) at 90°C for 16 hours to give methyl 3-hydroxy-13-methyltetradecanoate (**1**). The structure of N-4909 was thus identified as a subcomponent of isohalobacillin with a 3-oxy-13-methyltetradecanoyl moiety in the molecule. Although the absolute configurations of the amino acid residues in

Fig. 1. Structure of N-4909.

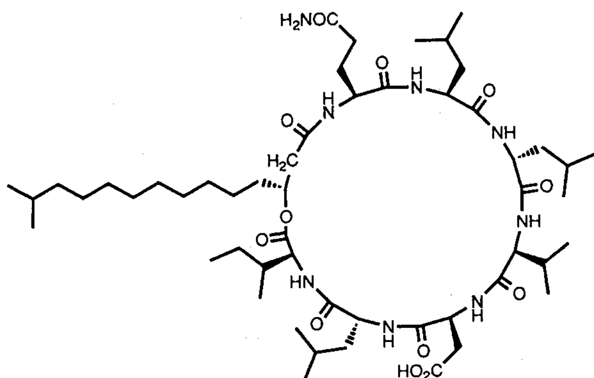
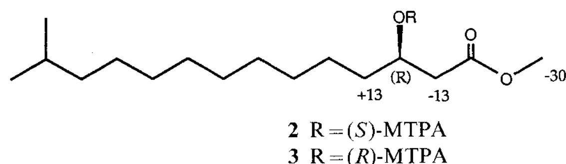


Table 1. Physico-chemical properties of N-4909.

Appearance	White powder
$[\alpha]_D^{27}$	−11.2° (*c* 0.4, MeOH)
Molecular formula	$C_{53}H_{94}N_8O_{12}$
HRFAB-MS $(M+H)^+$	
Found:	1035.7040
Calcd:	1035.7069
IR (KBr) cm^{-1}	3290, 2958, 2928, 2870, 1750, 1658, 1528, 1468, 1387, 1260, 1199, 1025, 796

Fig. 2. $\Delta\delta$ values ($\Delta\delta = \delta_S - \delta_R$ in hertz at 400 MHz) obtained for MTPA esters **2** and **3**.



2 R=(*S*)-MTPA
3 R=(*R*)-MTPA

Table 2. Effects of N-4909 on the secretion of apolipoproteins by Hep G2 cells.

N-4909 (μM)	Secreted apolipoproteins (ng/mg cell protein /7 hours)		
	apo E	apo A1	apo B
0	177±11	1223±50	1167±54
0.2	340±2* (192)	1373±91 (112)	1073±211 (92)
1.0	516±112* (292)	1520±137* (124)	750±148* (64)
5.0	734±65* (414)	1787±12* (146)	250±8* (21)

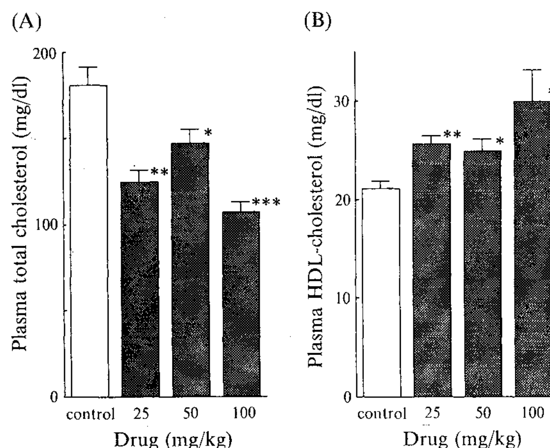
Hep G2 cells (obtained from Riken Cell Bank, Tsukuba) were seeded into 16-mm diameter dishes (1×10^5 cells/ml of DULBECCO's modified EAGLE's medium (DMEM) with 10% fetal bovine serum) and cultured for 3 days. The medium was replaced by serum-free DMEM and cells were further incubated for 24 hours. The medium was removed, and cells received serum-free DMEM containing several concentrations of N-4909 and were cultured for 7 hours. At the end of incubation, the cells and cultured medium were collected separately. Cells in each dish were dissolved in 0.1 N NaOH and cell protein was measured. Apo E, A1 and B levels in the medium were measured by enzyme-linked immunosorbent assay composed of commercially available antibodies, respectively. The values are mean±SD; $n=3$. Values in parentheses represent % of each control value.

* Significantly different from each control value by Student's t-test ($P<0.05$).

isohalobacillin had been determined, the stereochemistry of the 3-oxy-13-methyltetradecanoyl group remained unknown. Therefore, we have determined the absolute configuration of the 3-oxy-13-methyltetradecanoyl moiety by MOSHER's method[7]. Compound **1** was separately treated with (*S*)- and (*R*)-2-methoxy-2-phenyl-2-(trifluoromethyl)acetyl (MTPA) chlorides to yield (*S*)- and (*R*)-MTPA esters **2** and **3**. The ^{1}H NMR data of these esters revealed that the chemical shifts of corresponding protons were significantly and systematically different between 2 and 3 (Fig. 2). These data indicated the *R* configuration at the position 3 of the 3-oxy-13-methyltetradecanoyl moiety[7].

The secreted levels of apo E and other major apolipoproteins, apolipoprotein A1 (apo A1) and apo B from Hep G2 cells treated with N-4909 are summarized in Table 2. N-4909 stimulated apo E secretion in a dose-dependent manner. In the presence of 5.0 μM of the

Fig. 3. Effects of N-4909 on plasma total cholesterol (A) and HDL-cholesterol (B) levels in cholesterol diet-fed rats.



Male Sprague-Dawley rats were fed chow supplemented with 1% cholesterol and 0.5% cholic acid. All rats received 15 g of food daily and water *ad libitum* for 3 days. N-4909 was suspended in 0.5% methylcellulose and orally administered once a day for 3 days. On day 4, a blood sample was collected and both the total cholesterol and HDL-cholesterol levels in the plasma were determined by an enzymatic method using a commercially available kit (Wako Pure chemical Industries, Ltd., Osaka). Data are expressed as mean±SE of 5 rats.

$^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, as compared with the control group by Student's t-test.

compound, the apo E level in the medium was increased by 4.1 fold relative to the control. It also increased the apo A1 secretion in a dose-dependent manner. Secretion of apo A1 was less weakly stimulated than that of apo E by N-4909. Unlike apo E and apo A1, the apo B secretion into the medium from Hep G2 cells was suppressed by N-4909. This result implies that the compound suppresses the secretion of VLDL particles from Hep G2 cells, because most of the apo B exists in the fraction of VLDL and LDL in the cultured medium of Hep G2 cells[8].

The effects of N-4909 on nonspecific protein synthesis and secretion were investigated in Hep G2 cells. After preincubation with or without N-4909 for 1 hour, the cells were cultured in the medium supplemented with [^{3}H]-leucine for 7 hours. Under these conditions, N-4909 at 1.0 and 5.0 μM did not cause any significant alteration in the radioactivities of trichloroacetic acid-precipitable proteins in the cell and medium (data not shown). Thus, N-4909 was confirmed to stimulate selectively apo E secretion without affecting total protein synthesis and secretion.

The *in vitro* results described above suggested the possibility that N-4909 may show hypolipidemic activity *in vivo*. Consequently the ability of N-4909 to decrease the plasma cholesterol levels in cholesterol diet-fed rats was investigated. As shown in Fig. 3, oral administration of N-4909 at the doses of 25, 50 and 100 mg/kg/day significantly decreased the plasma total-cholesterol levels.

On the other hand, the high density lipoprotein (HDL)-cholesterol levels were significantly increased in the N-4909 treated groups. HDL-cholesterol elevation by hypolipidemic agents is highly desirable, since plasma HDL level is inversely correlated with the risk of coronary artery disease.

Several compounds such as 12-*O*-tetradecanoylphorbol-13-acetate, *N*-acetyl-leucyl-leucyl-norleucinal and HDL have been reported to stimulate apo E secretion from cultured cells[9~11]. However, no compound stimulating apo E secretion *in vitro* has been reported to show hypolipidemic activity *in vivo*. The present paper describes the first finding that a potent stimulator of apo E secretion from cultured cells decreased the plasma cholesterol levels in hyperlipidemic rats.

Although, the detailed mechanism of N-4909 on hypolipidemic activity in rats remains unknown, it seems very likely that N-4909 decreases the plasma cholesterol levels in hyperlipidemic rats through stimulating apo E secretion and suppressing the hepatic VLDL secretion. However, ACAT inhibition in the small intestine may be partly responsible for the decrease in plasma cholesterol in rats administered N-4909, because various ACAT inhibitors have been demonstrated to decrease plasma cholesterol levels[12]. Further study is necessary to clarify the mechanism of N-4909 on the hypolipidemic activity.

Experimental

Preparation of Methyl 3-Hydroxy-13-methyltetradecanoate (**1**)

N-4909 (30 mg) was dissolved in 3 ml of 12 N-HCl - methanol (3 : 7). After heating at 90°C for 24 hours, the reaction mixture was concentrated *in vacuo* and the resulted aqueous solution was extracted with *n*-hexane. The organic layer was washed, dried and evaporated to give an oily residue. This material was applied to preparative TLC, [*n*-hexane - diethyl ether - acetic acid (80 : 30 : 1)] to afford methyl 3-hydroxy-13-methyltetradecanoate (**1**) (4 mg).

Compound **1**: $[\alpha]_D^{25}$ +7° (*c* 0.5, MeOH); SI-MS *m/z* 273 $(M+H)^+$; ^1H NMR (400 MHz, $CDCl_3$) δ 4.01 (1H, m, *CH*OH), 3.72 (3H, s, CH_3O), 2.52 (1H, dd, J=2.9, 16.3 Hz, CH_2CO_2), 2.41 (1H, dd, J=9.3, 16.3 Hz, CH_2CO_2), 1.13~1.59 (19H, m), 0.86 (6H, d, J=6.8, $(CH_3)_2CH$).

Preparation of **2**

To a solution of **1** (2 mg) in dichloromethane (0.5 ml), (*S*)-MTPA chloride (5 µl) and 4-dimethylaminopyridine (6 mg) were added. After standing at room temperature for 15 hours, 3-[(dimethylamino)propyl]amine (4 µl) was added to the reaction mixture. The solvent was evaporated, and the residue was applied to preparative TLC [*n*-hexane - diethyl ether - acetic acid (80 : 30 : 1)] to give **2** (1 mg).

Compound **2**: $[\alpha]_D^{25}$ −20° (*c* 0.1, MeOH); SI-MS *m/z* 489 $(M+H)^+$; ^1H NMR (400 MHz, $CDCl_3$) δ 7.50 (2H, m, Ph), 7.38 (3H, m, Ph), 5.46 (1H, m, *CH*(OMTPA)), 3.57 (3H, s, CO_2CH_3), 3.51 (3H, d, J=1.0 Hz, CH_3O), 2.63 (1H, dd, J=7.8, 16.1 Hz, CH_2CO_2), 2.55 (1H, dd, J=4.9, 16.1 Hz, CH_2CO_2), 1.64 (2H, m, $CH_2CH(OCH_3)$), 1.49 (1H, m, $(CH_3)_2CH$), 1.10~1.29 (16H, m), 0.84 (6H, d, J=6.4, $(CH_3)_2CH$).

Preparation of **3**

To a solution of **1** (2 mg) in dichloromethane (0.5 ml), (*R*)-MTPA chloride (5 µl) and 4-dimethylaminopyridine (6 mg) were added. The reaction mixture was treated in the same manner as for the preparation of **2** to give **3** (1 mg).

Compound **3**: $[\alpha]_D^{25}$ −170° (*c* 0.1, MeOH); SI-MS *m/z* 489 $(M+H)^+$; ^1H NMR (400 MHz, $CDCl_3$) δ 7.51 (2H, m, Ph), 7.37 (3H, m, Ph), 5.46 (1H, m, *CH*(OMTPA)), 3.64 (3H, s, CO_2CH_3), 3.53 (3H, d, J=1.0 Hz, CH_3O), 2.68 (1H, dd, J=8.3, 16.1 Hz, CH_2CO_2), 2.59 (1H, dd, J=4.4, 16.1 Hz, CH_2CO_2), 1.60 (2H, m, $CH_2CH(OCH_3)$), 1.49 (1H, m, $(CH_3)_2CH$), 1.10~1.30 (16H, m), 0.84 (6H, d, J=6.4, $(CH_3)_2CH$).

Shigeru Hiramoto
Nobuhiro Kinoshita
Shigeo Hatanaka
Haruo Seto†

Pharmacology and Biochemistry Laboratory,
Nisshin Flour Milling Co., Ltd.,
5-3-1 Tsurugaoka, Ohi-machi,
Irumagun, Saitama 356, Japan
† Institute of Molecular and Cellular Biosciences,
The University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan

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